収穫後の‘蓮台寺'カキ果実におけるACC合成酵素およびACC酸化酵素の遺伝子発現

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Expression of 1-Aminocyclopropane-1-Carboxylate Synthase and 1-Aminocyclopropane-1-Carboxylate Oxidase Genes during Ripening in 'Rendaiji' Persimmon Fruit

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'Rendaiji' persimmon (*Diospyros kaki* Thunb.) is a pollination variant astringent type persimmon that is characterized by low levels of the ethylene production rate during ripening. However, a serious problem in postharvest handling is that mature fruits soften quite rapidly accompanied with increased ethylene production after treatments to remove astringency. In this study, we investigated changes in ethylene biosynthesis, by following the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC), as well as ACC synthase (ACS) and ACC oxidase (ACO) activities and the expression of their respective genes in relation to the process and mechanism of fruit ripening. The response by persimmon fruits to 1-methylcyclopropene (1-MCP) treatments during postharvest ripening was also studied. Ripening was accompanied by relatively low levels of the ethylene production rate, concomitant with a decrease in firmness, especially after exposure to a deastringency treatment by using ethanol vapor. Postharvest application of 1-MCP, on the other hand, did not suppress ethylene production rates to the expected level, but it lowered the accumulation of ACC and the activities of ACC synthase and ACC oxidase. However, the degree of inhibition was higher in ACC synthase, which would imply that the step catalyzed by this enzyme is more subject to regulation by ethylene in this fruit. Comparing gene expression patterns in control and 1-MCP treated fruit by using quantitative RT (Real-Time)-PCR showed that ethylene biosynthesis associated with rapid ripening in 'Rendaiji' was accompanied by the expression of *DK-ACS1*, *DK-ACS2*, *DK-ACO1*, and *DK-ACO2* genes. The expression of *DK-ACS3*, however, was not induced.

Key Words: *Diospyros kaki* Thunb., fruit maturity, 1-methylcyclopropene, ripening, softening.

Introduction

'Rendaiji,' a pollination variant astringent (PVA) persimmon is considered a natural treasure of Ise City in Mie Prefecture, Japan. The fruit is typically roundish-oblate spheroid with a relatively small calyx (Fig. 1). However, based on the shape, the fruit can further be divided into four types, namely; (a) 'Zairaikei'—fruit is small with few side and diagonal indentations and has few seeds; (b) 'Ohirakei-kobangata'—fruit is moderately large with few side and diagonal indentations; (c) 'Ohirakei-kikugata'—fruit is large and compressed with many diagonal indentations; and (d) 'Ohirakei-hesogata'—large fruit with numerous side indentations, the fruit is always double (or fused). 'Rendaiji' has a long harvest season that usually extends from the middle of September until early November (Hattori, 1994).

Like other PVA cultivars, such as 'Rojo Brillante' (Salvador et al., 2004), 'Rendaiji' is highly astringent during the immature stage. Similarly, astringency is lost only after the fruit becomes overripe which is concomitant with softening of the flesh. In Ise City, 'Rendaiji' growers usually subject the fruit to 75–80% CO_2 gas for 18–24 h at 18–22°C to remove its astringency (Hattori, 1994) but this treatment also promotes rapid flesh softening. Although the fruit has excellent eating quality, its high susceptibility to rapid softening after harvest has greatly limited its commercial life and eating quality.

According to our recent studies (Ortiz et al., 2005a, b), the rate of softening in 'Rendaiji' was rapid, such that as much as 8% of fruits reached softening index III (very soft) (Iwata et al., 1969) at the end of a typical 5-day deastringency treatment with ethanol vapor. In addition, the softening rate was accelerated further during storage at 20°C. Treatment with 1-MCP, a potent inhibitor of ethylene action (Sisler and Serek, 1997), in these fruit, however, significantly extended shelf-life and

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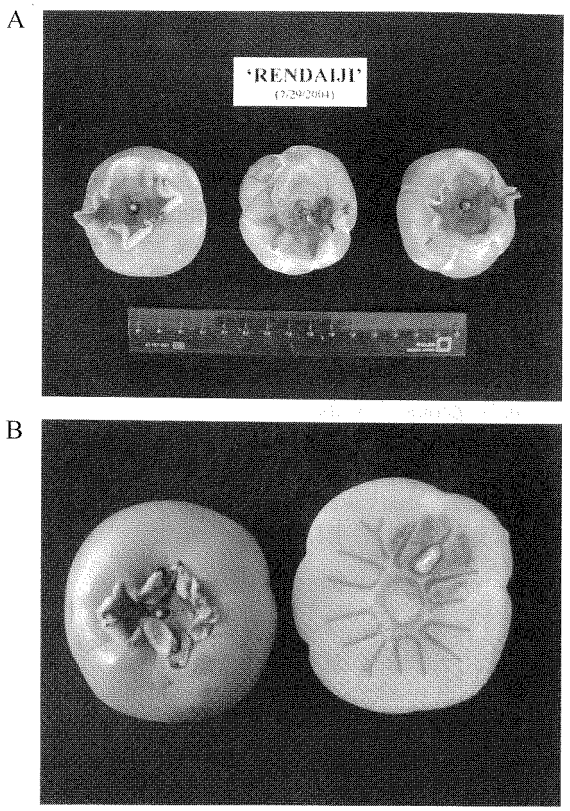

Fig. 1. 'Rendaiji' persimmon fruit. A: detached fruit at one month after full bloom, B: commercially mature fruit.

suppressed the activity of some cell-wall degrading enzymes. This suggests that the regulation of ethylene necessary for the induction of ripening had been altered.

Softening, as well as other changes associated with ripening in fruit, are induced and coordinated to some extent, by changes in the expression of genes that are involved in ethylene biosynthesis and changes in ethylene production. In persimmon fruit, the onset of the climacteric phase often triggers rapid softening (Harima et al., 2003). However, unlike other climacteric fruit, persimmons produce only small amounts of ethylene during the period of ripening (Wills et al., 1989), compared to the copious amounts produced at younger stages (Itamura, 1986; Itamura et al., 1997; Nakano et al., 2003; Takata, 1983).

Characterization and cloning of the genes encoding ACC synthase and ACC oxidase, the major enzymes of the ethylene biosynthetic pathway, in 'Hiratanenashi' and 'Tonewase' persimmon revealed that both enzymes may be developmentally regulated and spatially coordinated (Nakano et al., 2002, 2003). In these studies, softening in both young and mature persimmon fruit has been attributed to the action of the ethylene that is produced in the calyx, in response to water stress through the activated expression of *DK-ACS2*, which in turn, induces an autocatalytic ethylene production in the mesocarp. However, the regulation of fruit maturation and ripening by ethylene in 'Rendaiji' fruit remains to be elucidated. Moreover, an understanding of the regulation of the key enzymes involved in their synthesis and corresponding gene expression would provide an essential tool in the design of appropriate postharvest handling protocols, such as enhancing the storage potential and final fruit quality.

In this study, we used RT (Real Time) PCR to investigate changes in ethylene biosynthesis, ACC and MACC accumulation, ACC oxidase, ACC synthase activities, and the expression of their genes in mature 'Rendaiji' persimmon fruit harvested and stored at 20°C. We also investigated the effect of postharvest application of 1-MCP, a potent inhibitor of ethylene action, on some ripening-related changes.

Materials and Methods

Plant material and treatment

Mature 'Rendaiji' persimmons were harvested from the Science and Technology Promotion Center, Mie Prefecture, Japan on October 8, 2003 and immediately transported to the Laboratory of Pomology, University of Tsukuba for experimentation. Ethanol vapor treatment consisted of dipping fruits into a 35% ethanol solution, and then enclosing them in 0.03 mm thick polyethylene bags. The samples were stored in corrugated boxes for 5 days at 20°C. Subsequently, 1-MCP was applied by placing fruits inside a 12-L glass desiccator together with a petri dish that contained the amount of Ethylbloc® (Rohm and Hass, Japan) needed to generate the required volume of 1-MCP ($20\ \mu L \cdot L^{-1}$). Water was then added to the dish to release the 1-MCP gas, and the container was immediately closed and sealed for 24 h. Control fruits were placed in similar containers under identical conditions but without 1-MCP.

Determination of ethylene biosynthesis

Ethylene production by whole fruits enclosed in air-tight containers for 1 h was determined by withdrawing a 1-mL headspace gas sample and injecting it into a gas chromatograph (model GC 18A Shimadzu, Kyoto) equipped with a Porapak Q column and flame ionization detector. Ethylene was sampled by withdrawing 1 mL of headspace gas after 10 min incubation at 20°C.

ACC and MACC content, ACC oxidase, and ACC synthase activity

Tissues, obtained from fruit before the deastringency treatment, were extracted by using the method of Itamura et al. (1990), followed by ACC quantification according to Lizada and Yang (1979). MACC was measured based on the method described by Hoffman et al. (1982). ACC synthase (ACS) and in vitro ACC oxidase (ACO) activities in persimmon fruit tissue were measured at different times before and after treatment with ethanol and 1-MCP by using the method described by Zhang et al. (2003) and Kato et al. (2002), respectively.

RNA extraction and preparation of cDNA

Total RNA was isolated from the pulp of persimmon fruits by the method of Loulakakis et al. (1996), as modified by Mori et al. (2004) by using an extraction buffer that consisted of 200 mM Tris-HCl (pH 8.5), 300 mM LiCl, 1.5% lithium dodecyl sulfate, 1% sodium deoxycholate, and 1% EDTA.

RT-PCR and Real-Time quantitative RT-PCR

One microgram of total RNA was treated with RNase-free DNaseI and reverse transcribed by using the Superscript II RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was diluted at a concentration of 1:80 and used for PCR. The PCR amplification was performed with oligonucleotides specific for each gene and actin cDNAs by using the primers for *DK-ACS1*, *DK-ACS2*, *DK-ACS3*, *DK-ACO1*, *DK-ACO2*, and *DK-Actin* (Table 1).

Investigation on the expression profiles to evaluate the levels of genes involved in ethylene biosynthesis was carried out by using quantitative RT-PCR (Stratagene), a method that relies on real-time monitoring of the release of a fluorescent reporter dye (SYBR-Green I) as the PCR product accumulates in the reaction (Bustin, 2000). The cDNA was amplified with a Real-Time RT-PCR machine (MX3000P™, Stratagene, La Jolla, CA, USA). Amplification of actin cDNA under identical conditions was also conducted as an internal control to normalize the levels of cDNA as follows: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 57°C, 60 s at 72°C, and 15 s at 80°C. The cycle threshold (C_t) value for each PCR reaction was calculated. After completion of the amplification steps, the melting curve was determined for each analysis, followed by electrophoresis of the PCR products to confirm the specific amplification of each Q-PCR.

The sequences of the two ACO genes, *DK-ACO1* (AB073008) and *DK-ACO2* (AB073009), as well as the three ACS genes *DK-ACS1* (AB073005), *DK-ACS2* (AB073006), and *DK-ACS3* (AB073007) that were used in this study have been confirmed by cloning the RT-PCR products that were compared with database sequences by using the BLAST program.

Results

Ethylene evolution was 0.05 $nL \cdot g^{-1} \cdot h^{-1}$ in fruits harvested at maturity (October 9) (Table 2). After treatment with ethanol to remove astringency, the production rate decreased to undetectable levels and increased slowly during storage, reached a peak at day 16, and declined thereafter (data not shown). Patterns displayed for 1-MCP-treated fruits were similar with the

Table 1. Oligonucleotide primers used for the amplification of cDNAs by Real-Time RT-PCR.

Name	Position[z]	Accession number	DNA Sequence
DK-ACS1	F	AB073005	CCACTGCGACGAACCGGGTTGG
	R		TCAGGCTCGCGGCCATCGAACG
DK-ACS2	F	AB073006	AGAATCCGGACGTTCGTGGATGA
	R		AAGCATAGGGGAGTGAGGCGACAAC
DK-ACS3	F	AB073007	CTCAACGTCTCGCCGGGAGTCTCTT
	R		GAGGGGACATCATGGCAATGTCATC
DK-ACO1	F	AB073008	TGGCAATGATGCTGTTATCTATC
	R		CGAACTATTACAAATAACATGTGTC
DK-ACO2	F	AB073009	CAGCGACGCAGTGATTTATCCAG
	R		CAGAGGGCTTGGCTTAGACTGTGGC
DK-Actin	F	AB219402	GGTGATGGGGTGAGTCACACTGTACC
	R		GGTGATGGGGTGAGTCACACTG

[z] F and R denote forward and reverse primers, respectively.

Table 2. Effect of deastringency treatment with ethanol and 1-MCP on the rates of ethylene production, ACC content, MACC content, ACC oxidase and ACC synthase activity in harvested fruits.

Treatment time	Ethylene		ACC		MACC		ACC oxidase		ACC synthase	
	Control	1-MCP	Control	1-MCP	Control	1-MCP	Control	1-MCP	Control	1-MCP
days	$nL \cdot g^{-1} \cdot h^{-1}$		$nmol \cdot g^{-1}$		$nmol \cdot g^{-1}$		$nmol\ C_2H_4 \cdot g^{-1} \cdot h^{-1}$		$nmol\ C_2H_4 \cdot g^{-1} \cdot h^{-1}$	
0	0.05 ± 0.01		0.57 ± 0.43		1.33 ± 0.11		8.07 ± 1.2		0.12 ± 0.10	
6	trace	trace	1.13 ± 0.29	0.75 ± 0.58	0.03 ± 0.01	0.33 ± 0.01	73.29 ± 1.7	3.54 ± 0.37	0.35 ± 0.08	0.068 ± 0.04
11	0.03 ± 0.02	0.01 ± 0.01	4.13 ± 0.46	0.01 ± 0.00	0.29 ± 0.19	trace	79.17 ± 10	18.88 ± 3.32	1.12 ± 0.09	0.099 ± 0.04
16	0.08 ± 0.01	0.05 ± 0.01	0.78 ± 0.02	trace	0.33 ± 0.21	trace	trace	89.90 ± 2.33	0.11 ± 0.02	trace

The values are the means ± SE of three replications.

control except that the treated fruits initiated ethylene production one day later than the control (data not shown). ACC content in control fruits increased from 0.57 at harvest to 4.13 nmol·g^{-1} at day 11 while that in the 1-MCP treated sample decreased to 0.01 nmol·g^{-1} with no recovery even after the climacteric peak occurred (Table 2). The increases in ACC synthase as well as ACC oxidase activity were also inhibited to a large extent by 1-MCP, however, a surge in ACC oxidase activity was observed on day 16.

Results of quantitative real time RT-PCR revealed that *DK-ACS2*, which was low in the pulp after deastringency treatment reached a peak at day 11 parallel with *DK-ACS1*; it declined afterwards (Fig. 2A, B). 1-MCP, on the other hand, effectively suppressed the expression of both ACS genes (Fig. 2A, B), concomitant with little or no recovery in ACS enzyme activity even after the climacteric peak at day 16 (Table 2).

Contrarily, *DK-ACS3* gene expression was relatively low but was consistent in the pulp of commercially mature fruits (Fig. 2C). Accumulation of *DK-ACS3* was also detected even in fruits treated with 1-MCP (Fig. 2C).

However, expression of genes encoding *DK-ACO1* and *DK-ACO2* changed very little at harvest and after the deastringency treatment with ethanol (Fig. 3A, B). A peak in transcript levels for both genes was detected at day 11, with values relatively higher in *DK-ACO2*, followed by a decrease in its level of expression at day 16. In contrast, 1-MCP treatment did not suppress the expression level for both genes at day 6 or immediately following treatment (Fig. 3A, B). However, an inhibition, although not complete, was evident at day 11, especially in *DK-ACO2* where levels were almost ten-fold lower than those of the control.

Discussion

Ethylene evolution in mature 'Rendaiji' persimmon fruits after harvest and deastringency treatment with ethanol was characterized by a drop in ethylene production and a slow increase starting at day 8 (data not shown) to reach a peak at day 16. Concomitant with

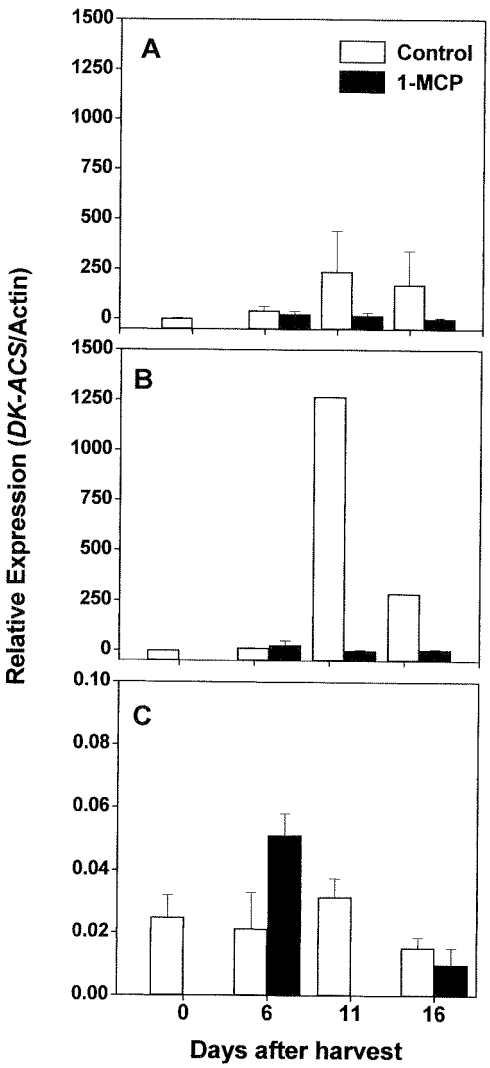

Fig. 2. Real-Time RT-PCR analysis of the expression of *DK-ACS1* (A), *DK-ACS2* (B), and *DK-ACS3* (C) genes in the pulp of commercially mature persimmon fruit treated with or without 1-MCP after deastringency treatment with ethanol vapor. Real-Time RT-PCR amplification of β-actin was used to normalize expression of the genes under identical conditions. Vertical bars represent SE of the means of three replications.

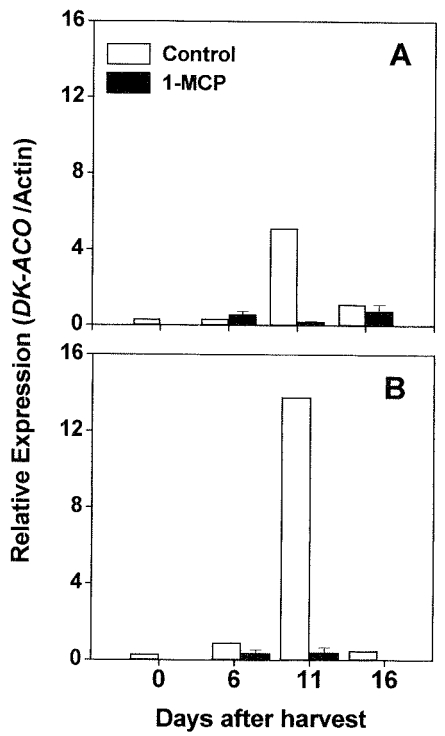

Fig. 3. Real-Time RT-PCR analysis of the expression of *DK-ACO1* (A), and *DK-ACO2* (B) genes in the pulp of commercially mature persimmon fruit treated with or without 1-MCP after deastringency treatment with ethanol vapor. Real-Time RT-PCR amplification of β-actin was used to normalize expression of the genes under identical conditions. Vertical bars represent SE of the means of three replications.

the changes in ethylene production rate, firmness in these fruits dropped from 2.82 kg at harvest to 2.54 kg after treatment with ethanol (data not shown). Postharvest application of 1-MCP, however, did not suppress ethylene production rates significantly (Table 2) as measured by using headspace gas analysis, but it suppressed the activities of both enzymes, especially ACC synthase. The result would imply that ACC synthase but not ACC oxidase is more subject to regulation by ethylene in mature 'Rendaiji' persimmon fruits.

Since ACS is encoded by a multigene family (Lelievre et al., 1997), differential regulation of members of the ACS gene family during fruit development and ripening has been reported for many fruit species including persimmon (Nakano et al., 2003). In fruit harvested at maturity, a weak expression of *DK-ACS1* and *DK-ACS2* was detected a day later, which gradually increased many folds as ripening progressed (Fig. 2A, B). In ripening 'Hiratanenashi' persimmon fruit, ethylene production has been attributed to the accumulation of *DK-ACS1* mRNA (Nakano et al., 2003) and *MA-ACS1* in banana (Liu et al., 1999). In general, ACS activity in harvested fruit correlates with ethylene evolution. Consistent with the levels of ACS mRNA, ACC synthase activity and ACC accumulation were suppressed in fruit that had been exposed to 1-MCP (Fig. 2, Table 2). In these fruits, inhibition of expression by 1-MCP was almost absolute in *DK-ACS2* and *DK-ACS1*. In contrast, the expression of *DK-ACS3* persisted throughout ripening and was not influenced by treatment with 1-MCP (Fig. 2C) which suggests that the expression of this gene is regulated in an ethylene-independent manner. However, the expression of this gene was not detected in 'Tonewase' persimmon fruits that were subjected to either low or high humidity or in the presence of 1-MCP (Nakano et al., 2002).

Expression of both ACO genes, *DK-ACO1* and *DK-ACO2*, in control and 1-MCP-treated fruits were detectable throughout the experimental period (Fig. 3). ACO gene expression is regulated by ethylene in many climacteric fruits, such as tomato (Barry et al., 1996) and apples (Dong et al., 1992). In tomato, *LE-ACO1* was expressed mainly in ripening fruit (Nakatsuka et al., 1998), whereas *PP-ACO1* became abundant in ripening peaches (Mathooko et al., 2001), mume (Mita et al., 1997), and 'Tonewase' persimmon (Nakano et al., 2002). 1-MCP application, caused a transient suppression in the activity of ACC oxidase whereby levels almost dropped twentyfold, but a dramatic increase in activity occurred on day 16, which far exceeded levels in control fruits (Table 2). This surge in activity, however, did not coincide with the levels of gene expression in both *DK-ACO1* and *DK-ACO2* (Fig. 3A, B). This discrepancy could be partly explained by the presence of other regulatory factors that control the processes of transcription and translation and the activity of the specific enzyme. The trend observed at day 11 in control fruit was that the low levels of ethylene evolution did not coincide with the relatively high ACC content and ACO activity. In tomato, this phenomenon was attributed to an apparent differing sensitivity of ACO isomers to both in vitro and in vivo ACO assays, as observed by Binonde et al. (1998), who also reported that they exhibited different K_m values for ACC.

Based on our results, ethylene production in ripening 'Rendaiji' persimmon fruit can be characterized as follows: (a) there is an initial increase in ethylene production after harvest coupled with basal levels of ACO and ACS enzyme activity as well as the expression of their respective genes in the pulp; (b) ethylene, even in small amounts can be regulated by the different members of the ACS gene family; (c) *DK-ACS1* and *DK-ACS2* are regulated by ethylene but not *DK-ACS3*, at least in mature 'Rendaiji' persimmon fruit; and (d) application of 1-MCP failed to suppress *DK-ACS3* expression but exerted its effect mostly on the expression of *DK-ACS1* and *DK-ACS2*.

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収穫後の‘蓮台寺’カキ果実におけるACC合成酵素およびACC酸化酵素の遺伝子発現

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不完全渋ガキの‘蓮台寺’は収穫熟度の果実ではエチレン生成量が低いが，脱渋処理後エチレンを発生し，収穫後の取り扱いの中で急速に軟化することが問題である．本研究では，追熟過程の果実，およびエタノール脱渋後 1- メチルシクロプロペン（1-MCP）処理をした果実におけるエチレン生成，エチレン生合成酵素活性および遺伝子発現を解析し，エチレン生成制御について検討した．その結果，エタノール脱渋後の果実ではエチレン量は比較的低いレベルであったが，エチレン生成量の増加は果実硬度の低下と一致していた．1-MCP 処理果では，エチレン生成量はそれほど抑制されなかったが，ACC 量，ACC 合成酵素および ACC 酸化酵素活性は明らかに低下した．その阻害の程度は ACC 合成酵素で著しかったことから，エチレンによる‘蓮台寺’果実の成熟制御には，本酵素が重要であることが示唆された．リアルタイム PCR を用いて 1-MCP 処理果および追熟果の成熟過程におけるエチレン生合成酵素遺伝子の発現量の変化を調べたところ，‘蓮台寺’果実では，追熟に伴ったエチレン生成により *DK-ACS1*, *DK-ACS2*, *DK-ACO1*, *DK-ACO2* が強く発現誘導されることにより追熟が急速に進行することが示唆された．一方，追熟果では *DK-ACS3* の発現はほとんど発現が認められなかった．